

Quantitation of collagen types I, III and V in tissue slices by capillary electrophoresis after cyanogen bromide solubilization

Zdeněk Deyl^{a,*}, Jana Novotná^b, Ivan Mikšík^a, Dita Jelínková^c, Marie Uhrová^c,
M. Suchánek^c

^a*Institute of Physiology, Academy of Sciences of the Czech Republic, Videňská 1083, CZ 142 20 Prague 4, Czech Republic*

^b*2nd Medical School Charles University, Dept. Med. Chem. Biochem., Plzeňská 221/130, CZ 150 00 Prague 5, Czech Republic*

^c*Department of Analytical Chemistry, Institute of Chemical Technology, Technická 1905, CZ 166 28 Prague 6, Czech Republic*

Abstract

A method for the determination of the proportions of major fiber-forming collagens (types I, III and V) in soft connective tissue was elaborated. The method is based on the release of insoluble collagen by CNBr with subsequent separation of the arising peptides. For routine application the peptides are separated by capillary electrophoresis (50 mM phosphate pH 2.5, 15 kV, 50°C, 70/60 cm×70 μm I.D. capillary with UV detection at 200 nm). Quantitation of collagen type I can be done either on the basis of spiking the sample with a peptide mixture obtained from a known amount of collagen type I, or by spiking the sample with an equimolar mixture of the two peptides [α_1 (I)CB₂ and α_1 (I)CB₄] (constituting a fused peak) along with α_1 (III)CB₂ and α_1 (V)CB₁. Compared to the previously published methods the procedure is faster and does not require isolation of marker peptides by tedious chromatographic procedures in a preceding preparatory step. Good results are obtained within a wide range of run buffer concentrations and applied voltages; conversely, intensive cleaning of the capillary after every three runs is recommended with a new capillary after 20–30 runs.

Keywords: Collagens

1. Introduction

Fiber-forming collagens represent a set of at least nine different polypeptide chains which constitute the molecular species of type I, III, V and XI collagens. Types I, III and V are codistributed in tissues [1,2] and there is a lot of information indicating that their relative proportions change during development [3–6], wound healing [7,8] and under some pathological conditions [9–11]. During the last few years evidence has accumulated about the existence of heterotypic collagen fibrils or fibres composed by copolymerization of different types of

collagen, particularly type I and II [12], type I and V [13], as well as all main fibril forming collagens (i.e. I, III and V) together [14]. It was also proposed that copolymerization (coaggregation) of different molecular species of collagen represents the mechanism which controls fiber diameter.

Collagen molecules of the most common species, collagen type I, are constituted of three polypeptide chains named α_1 (two chains) and α_2 . Individual constituting α -chains can undergo CNBr fragmentation. Owing to the limited amount of methionine residues a relatively simple peptide mixture arises. The nomenclature of these peptides refers to the parent polypeptide chain: α_1 constituting polypeptide chain yields a set of α_1 CNBr peptides (α_2 chain

*Corresponding author.

yields in analogy a set of α_2 CNBr peptides). The index, e.g. α_1 CB₁, identifies a particular peptide within the set. The number in parenthesis refers to the collagen type, e.g. α_1 (I)CB₁ means CNBr peptide of collagen type I. All of the peptides are present in individual parent polypeptide chains only once: this means that the molar ratio of any CNBr peptide arising from α_1 chain to any CNBr peptide arising from the α_2 chain must be 2:1 (because there are two α_1 chains and one α_2 chain per collagen type I molecule).

Quantitation of collagen proteins in tissues is routinely done by hydroxyproline assay [15]. Such an analysis, however, neglects the fact that more collagen species differing slightly in hydroxyproline content may be involved. Furthermore, the limit of detection of this assay frequently requires pooled samples, typically when analysing small tissue samples (e.g. pulmonary arteries). In order to be able to investigate individual arteries and reveal the inter-individual variability a method allowing collagen estimation in small arterial sections is needed. In addition, it is expected that not only the total amount of collagen may change in e.g. pulmonary hypertension but that the proportion of individual collagen types may also change. This underscores the need for a fast, reliable, rugged and sensitive method for determining the proportion of individual fibril/fiber-forming collagens in tissues.

The analysis of tissues for collagen types I and III has followed in the past three strategies. In the first strategy collagenous stroma is solubilized (after exhaustive extraction) by limited proteolysis with pepsin and subsequent separation of collagen α -chains by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Under reducing conditions and proper arrangement of the electrophoretic separation step all three α -chains involved, namely α_1 (I), α_2 (I) and α_1 (III) may be separated and quantitated. There are two difficulties involved with this approach. Firstly, as discussed by Miller et al. [16] it is hard to achieve a quantitative solubilization of collagens from most tissues and, secondly, quantitation of collagens after gel electrophoresis suffers from variability of staining intensity and incomplete resolution of the individual α -chains. If the analyses are based on recovery of collagen precipitates, large samples are evidently needed.

The other two approaches are similar in that they

involve solubilization of tissue collagens by CNBr; the difference is, however, in the way the liberated CNBr peptides are separated. In the older approach the arising mixture of CNBr peptides is separated by SDS–PAGE gel electrophoresis and the staining intensity of individual bands is compared with marker mixtures. Staining can be based on the affinity of peptidic material to the usual dyes (bromophenol blue, nigrosine, amido black, etc.), or on the antibody reactivity following transfer to nitrocellulose [17]. The main advantage here is that CNBr treatment of tissue collagens offers their quantitative solubilization and recovery. Conversely, the result of such a treatment is a large number of peptides which are difficult to separate in a single electrophoretic run. The relatively recent attempt to overcome the individual problems is based on *in vitro* labelling of the CNBr peptides with tritium, followed by a second CNBr digestion step and two-dimensional gel electrophoresis of the arising mixture [18] followed by a fluorographic evaluation of the arising two-dimensional peptide map.

An alternative to the final gel electrophoretic separation of the arising mixture was proposed by Miller et al. [16]. The collagens are likewise solubilized by CNBr digestion of the tissue, but the arising peptide mixture is subjected to two subsequent chromatographic steps, namely cation-exchange chromatography followed by a gel permeation procedure in a manner consistent with a relatively rapid resolution and quantitation of relatively low-molecular-mass marker peptides for each collagen. The marker peptides used for individual collagen species involved were α_1 (I)CB₂, α_1 (III)CB₂ and α_1 (V)CB₁. Quantitation of individual peptides was based on UV absorbance at 220 nm during the last chromatographic step (gel permeation). This approach adds to sensitivity and allows routine evaluation of collagen composition in tissues. The practical applicability of the method was demonstrated in the analyses of placental membrane and blood vessel walls specimens. It is claimed that quantitation of individual peptides as a function of UV absorbance in the final chromatographic step eliminates some uncertainties involved in quantitating the fragments after staining in polyacrylamide gels. It was also documented that the method allows detection and quantitation of as little as 2–3% of collagen type V in the mixture.

From the work of Miller et al. [16] it can be

concluded that direct quantitation by UV should be preferred in any advanced procedure for CNBr fragments quantitation. It is noticeable that in this paper [16] no reversed-phase separation of CNBr peptides of all the involved collagen species is mentioned, though such a technique has been described in 1980 by van der Rest et al. [19]. The separation is possible with Vydac TP 201 (30 nm pore size) with a linear acetonitrile–water gradient containing 0.01 M heptafluorobutyric acid. Resolution is relatively good for the peptides arising from a particular collagen species but unsatisfactory for a complex mixture. The remarkable fact about this separation is that individual peptides are eluted in the order of increasing molecular mass which is explained on the basis of the high internal homogeneity of collagen polypeptide chains.

All the above approaches are multistep protocols which complicates their application for routine analyses though admittedly the two step chromatographic procedure [16] appears the least time consuming and most easy to perform. In a recent report we have described the separation of collagen type I CNBr peptides by capillary electrophoresis under acidic conditions [17]. Though the separation of CNBr peptides under alkaline conditions is possible as well [20], it requires extremely dilute buffers with low buffering capacity which consequently means that good reproducibility of migration times can be obtained only with very careful control of separation conditions. In acid media (pH 2.5) using 100 mM phosphate buffer as background electrolyte and 50 cm capillary (50 μm I.D., 8 kV) good selectivity and migration time reproducibility were obtained. While in alkaline buffers separation of collagen CNBr peptides followed the Offord equation [21], in acid media the separation was very similar to the result of reversed-phase chromatography and the peptides (with the exception of the relatively small ones) were eluted according to their increasing molecular mass [17].

In the present communication we report separation of small-molecular-mass marker peptides for collagen types I, III and V by capillary electrophoresis. Both artificial mixtures as well as the application of this approach to small blood vessel wall collagen analysis was used. The marker peptides best suited for quantitation of individual collagen types proved nearly the same as used by Miller et al. [16] i.e.

$\alpha_1(\text{I})\text{CB}_2$ [in some cases also $\alpha_1(\text{I})\text{CB}_4$], $\alpha_1(\text{III})\text{CB}_2$ and $\alpha_1(\text{V})\text{CB}_1$ and $\alpha_2(\text{I})\text{CB}_4$.

2. Experimental

2.1. Capillary electrophoresis

All separations were done on a Spectra Phoresis 500 TSP-instrument, obtained from Watrex (Prague, Czech Republic) using PC 1000 software version 2.6. The capillary used was 70 cm long (63 cm to the detector) (75 μm I.D.), uncoated run at 15 kV if not specified otherwise (yielding 30–70 μA current depending on temperature). Separations were run with 20–100 mM phosphate buffers either purchased from Bio-Rad (Richmond, CA, USA, catalogue No 148-5010 with polymeric modifier) or laboratory prepared. Concerning the separation of marker peptides only small differences were seen between the two types of electrolytes used. Runs at 25°C and 50°C were performed to reveal the influence of denaturation of the large peptides present in the mixture and buffer concentration (20–100 mM buffers were used as specified in the text). Depending on the protein content in the sample 1–8 s hydrodynamic sample application (vacuum 10.3 kPa) was used. UV absorbance was detected at 200 nm.

2.2. Preparation of CNBr peptides

CNBr peptides were prepared from collagen type I (Sigma, St. Louis, MO, USA) or types III and V collagens prepared in the laboratory from rat tail tendons or respective tissues as specified in the text following limited pepsin digestion and selective salt precipitation according to established procedures [22]. These samples were treated with CNBr without chain separation in 70% formic acid as described by Scott and Veis [23]. This procedure yielded a considerable proportion of uncleaved peptides which emerged closely to the joint peak of $\alpha_1(\text{I})\text{CB}_7$ and $\alpha_1(\text{I})\text{CB}_8$; therefore in some experiments the reaction time was increased up to 24 h.

Peptide preparation directly from tissue slices followed generally the same procedure following incubation of the tissues in 0.2 M ammonium bicarbonate pH 7.0 containing 25% β -mercaptoethanol to reduce oxidized methionyl residues and

thereby to enhance CNBr cleavage [24]. Lyophilized CNBr peptide preparations from tissue samples were redissolved in Milli-Q water, centrifuged and lyophilized again, before analysis they were reconstituted either in formate buffer, 1% acetic acid or water at a concentration 400 $\mu\text{g/ml}$.

2.3. Isolation of marker peptides and proof of their identity

The liquid chromatography equipment was composed of two Model 6000A pumps, a Model 660 gradient programmer and Model U6K injector, all from Waters (Millipore, Bedford, MA, USA). Two types of columns were used.

2.3.1. Cation-exchange chromatography

For the isolation of $\alpha_1(\text{I})\text{CB}_2$, $\alpha_1(\text{III})\text{CB}_2$ and $\alpha_1(\text{V})\text{CB}_1$ peptides the initial peptide mixtures obtained by cleaving individual collagen types were chromatographed on a cation-exchange column (Mono S HR 5/5, Pharmacia, Uppsala, Sweden) in 20 mM (Na^+) sodium formate (pH 3.8 starting buffer) at a flow-rate 1.0 ml/min (column 35 \times 1.5 cm I.D.). For this chromatographic procedure peptides were dissolved in starting buffer and 0.5-ml volumes were injected. The column was run with the starting buffer for 10 min followed by a linear gradient of 0.4 M NaCl in the starting buffer to create the salt gradient (total running time was 40 min). The effluent was monitored at 200 nm and the peaks of interest were collected in 10-ml vials. The whole procedure followed that described by Miller et al. [16].

2.3.2. Reversed-phase chromatography

Isolation of the $\alpha_2(\text{I})\text{CB}_4$ peptide was done by reversed-phase chromatography as described by van der Rest et al. [19] and in our previous communication [17]. The column used was Vydac TP 201 column (Separations Group, Hesperia, CA, USA) (250 \times 4.6 mm I.D., 10 μm particle size, 30 nm pore size). Detection was done at 200 nm and the fraction eluting at 40–42 min was collected. Elution was done with a linear gradient 12.8–44.8% acetonitrile in water (v/v), starting and limiting eluent were 10 mM with respect to heptafluorobutyric acid (HFBA). The flow-rate applied was 1.0 ml/min. This pro-

cedure was also used for the separation of other peptides released after collagen type I collagen needed for peak identification in the capillary electrophoresis runs.

2.4. Isolation of collagen from peripheral pulmonary arteries

Segments of the pulmonary arteries (lateral branches of the third order) were cut into a small pieces, 20 mg of tissue (wet mass) was extracted by 15 volumes of 4 M guanidine chloride with 50 mM sodium acetate, pH 5.8, 48 h at 4°C, to remove noncollagenous proteins. After washing in distilled water the remaining tissue was pepsinized with 10 volumes of 2% pepsin (w/v) in 0.5 M acetic acid for 4 h at room temperature and 20 h at 4°C, centrifuged and lyophilized.

2.5. CNBr peptides extraction from polyacrylamide gel

CNBr peptides were extracted from polyacrylamide gel using two Amicon products (Amicon, a Grace Co., Beverly, MA, USA). Protein peptide was recovered from polyacrylamide gel with Micro-pure inserts and Microcon microconcentrators (Ultrafiltration protocol, Pub. 311). By this way the $\alpha_1(\text{I})\text{CB}_6$ peptide was obtained and identified in the capillary electrophoretic profiles (data not shown).

2.6. Chemicals

All chemicals used were either of analytical grade or highest available purity. Formic acid (88%) was obtained from Lachema (Brno, Czech Republic), collagen type I, pepsin (activity 1500–3500 U/mg protein), 2-mercaptoethanol, CNBr, guanidine hydrochloride and ammonium bicarbonate were products of Sigma. Sodium formate was purchased from Baker (Phillipsburg, NJ, USA). Phosphate buffer pH 2.5 was either purchased from Bio-Rad or prepared from sodium monophosphate (Lachema) with pH adjusted by phosphoric acid (Lachema). All solutions were prepared in Milli-Q water (Millipore).

3. Results

3.1. Exposition of the method

While capillary electrophoresis in alkaline buffers did not result in acceptable profiles of collagen CNBr peptides, separation in acidic buffers at pH 2.5 resulted in a complex set of peaks, varying considerably in their retention times and absorbance. Small-molecular-mass peptides were resolved particularly well (resolution ≥ 1) and their retention times showed S.D. values between 0.11 and 0.46 min depending on the experimental conditions (buffer concentration, temperature, voltage).

In general, it was observed that the experimental conditions may vary over a rather wide range with still acceptable results. We have used temperatures ranging between 25 and 50°C, buffer concentrations between 20 and 100 mM and voltages of 10–25 kV per 70 cm long capillary with very similar results.

A typical profile obtained with a commercial preparation of collagen type I (Bio-Rad buffer diluted 1:1 with Milli-Q water) is shown in Fig. 1; the run was performed in a commercially available buffer of 50 mM phosphate pH 2.5 at 15 kV with an polymer modifier claimed (by the manufacturer) to exhibit sieving properties. When however, the individual peptides were identified by spiking with purified CNBr fragments (see Section 2) it turned out that the increase of migration time with increasing molecular mass was valid for peptides of molecular mass larger than 13 500 (149 amino acid, AA, residues in collagen type I). Peptides having molecular mass less than 4600 behaved irregularly and this irregularity was even more pronounced when the mixture was spiked with small-molecular-mass peptides originating from type III and type V collagens $\alpha_1(\text{III})\text{CB}_2$ (40 AA residues) and $\alpha_1(\text{V})\text{CB}_1$ (54 residues). The profile seen in Fig. 1 is basically that of CNBr peptides of collagen type I spiked with $\alpha_2(\text{I})\text{CB}_1$ telopeptide, and the previously mentioned marker peptides of collagen type III and V. Spiking of the mixture with the $\alpha_1(\text{V})\text{CB}_1$ peptide was on the 5% level of total collagen, spiking with peptide $\alpha_1(\text{III})\text{CB}_2$ corresponded to 30% of collagen type III (these concentrations correspond to what may be found in natural samples). The separation in this buffer was relatively fast (the largest peptide

$\alpha_2(\text{I})\text{CB}_{3,5}$, 672 AA residues) emerged around 36 min. The run was done at 25°C and the separation factor of the small peptides (less than 149 AA residues) was always ≥ 1 (including the spiked ones) with the sole exception of $\alpha_1(\text{I})\text{CB}_5$ and $\alpha_2(\text{II})\text{CB}_2$ peptides, the resolution of which was incomplete. However, these peptides were not used as marker peptides for individual collagen type I polypeptide chains.

This run was done with peptides obtained under milder cleavage conditions (shorter CNBr reaction time) which resulted in a set of incompletely cleaved peaks emerging between 32 and 34 min running time.

The question which arose at this stage of our investigation was to what extent the separation is influenced by the presence of the sieving properties of this commercial background electrolyte (the nature of which was not disclosed by the producer). Therefore another experiment was done in pH 2.5 phosphate buffer prepared in the laboratory. The profile was very similar except that the $\alpha_2(\text{I})\text{CB}_{3,5}$ peptide did not emerge after 90 min running time. Therefore the voltage was increased to 20 kV per capillary and the result shown in Fig. 2 was obtained. Again a complete resolution of the low-molecular-mass peptides was obtained with the exception of the $\alpha_1(\text{I})\text{CB}_5$ and $\alpha_2(\text{I})\text{CB}_2$ peptides which in all our runs were difficult to separate. In addition the $\alpha_1(\text{I})\text{CB}_5$ and $\alpha_2(\text{I})\text{CB}_2$ were fused with the high-molecular-mass (~218–280 AA residues) peptide cluster. These latter two peptides were clearly resolved if the voltage was decreased, although causing a longer run time. Also under these conditions the order of the $\alpha_1(\text{I})\text{CB}_1$ and $\alpha_1(\text{V})\text{CB}_1$ peptides was reversed in comparison with the run performed with the polymer modifier containing background electrolyte (see Fig. 1). This was verified by running these two peptides in a mixture (data not shown). The region of low-molecular-mass peptides was followed by a cluster of peptides comprised of more than 200 AA residues. Because runs shown in Fig. 2 were done with peptides obtained after prolonged CNBr cleavage, no distinct peak of incomplete cleavage products after the dominant fused peak of large peptides was observed (cf. Fig. 1).

Because it is assumed (see also our previous communication [17]) that at least the large peptides

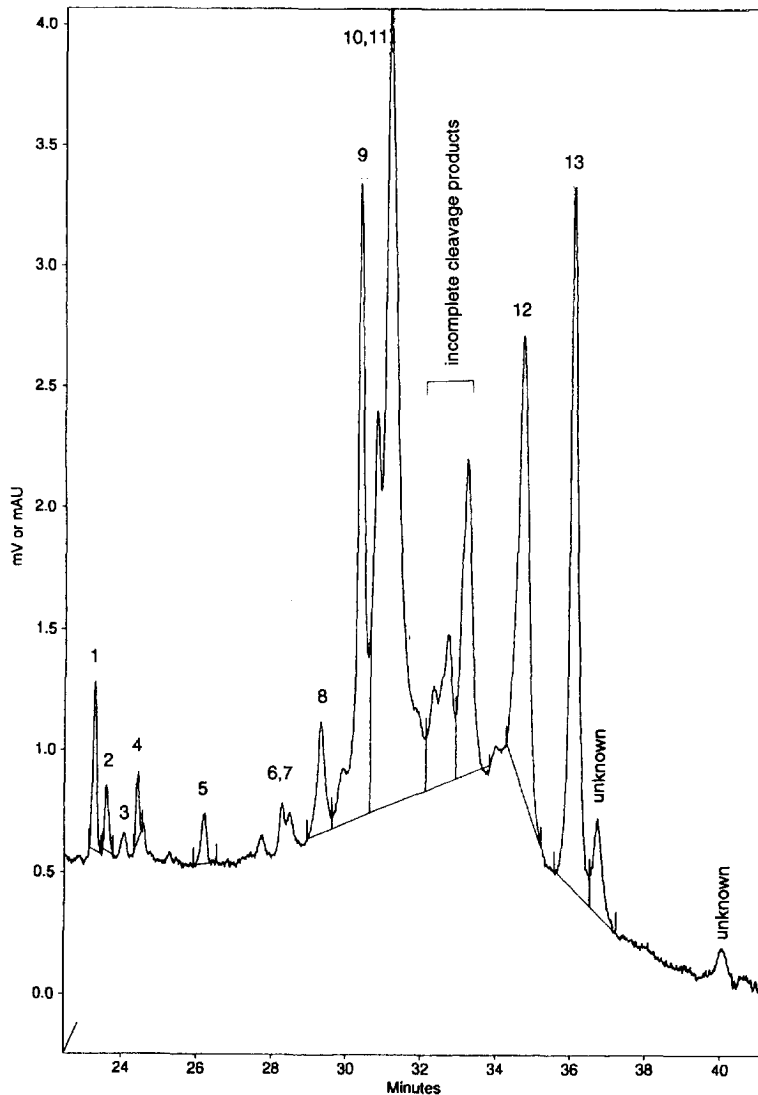


Fig. 1. Separation of collagen type I CNBr peptides obtained from a commercial preparation (Sigma) spiked with $\alpha_1(\text{V})\text{CB}_1$ and $\alpha_1(\text{III})\text{CB}_2$ as described in Section 2. Background electrolyte used was Bio-Rad pH 2.5 phosphate buffer (with polymeric modifier) diluted 1:1 with Milli-Q water; 15 kV, 75 cm \times 75 μm I.D. untreated capillary. Temperature 25°C, UV detection at 200 nm. Peak identification: 1 = $\alpha_1(\text{I})\text{CB}_2$, 2 = $\alpha_1(\text{I})\text{CB}_4$, 3 = $\alpha_1(\text{V})\text{CB}_1$, 4 = $\alpha_2(\text{I})\text{CB}_1$, 5 = $\alpha_1(\text{III})\text{CB}_2$, 6 = $\alpha_1(\text{I})\text{CB}_5$, 7 = $\alpha_2(\text{I})\text{CB}_2$, 8 = $\alpha_1(\text{I})\text{CB}_3$, 9 = $\alpha_1(\text{I})\text{CB}_6$, 10 = $\alpha_1(\text{I})\text{CB}_7$, 11 = $\alpha_1(\text{I})\text{CB}_8$, 12 = $\alpha_2(\text{I})\text{CB}_4$ and 13 = $\alpha_2(\text{I})\text{CB}_{3,5}$.

(containing more than 200 AA residues) are separated on the basis of their affinity to the capillary wall, we were interested to what extent the separation can be affected by running the electrophoresis under denaturation conditions. The sample was boiled for 1 min and the whole run was done at

50°C. Except the shortened run time, no difference in the overall patterns was observed (Fig. 2B).

The influence of the background electrolyte concentration (without polymeric buffer modifier) is seen in Fig. 3. Only a slight increase in migration time was observed when running the separation in 75

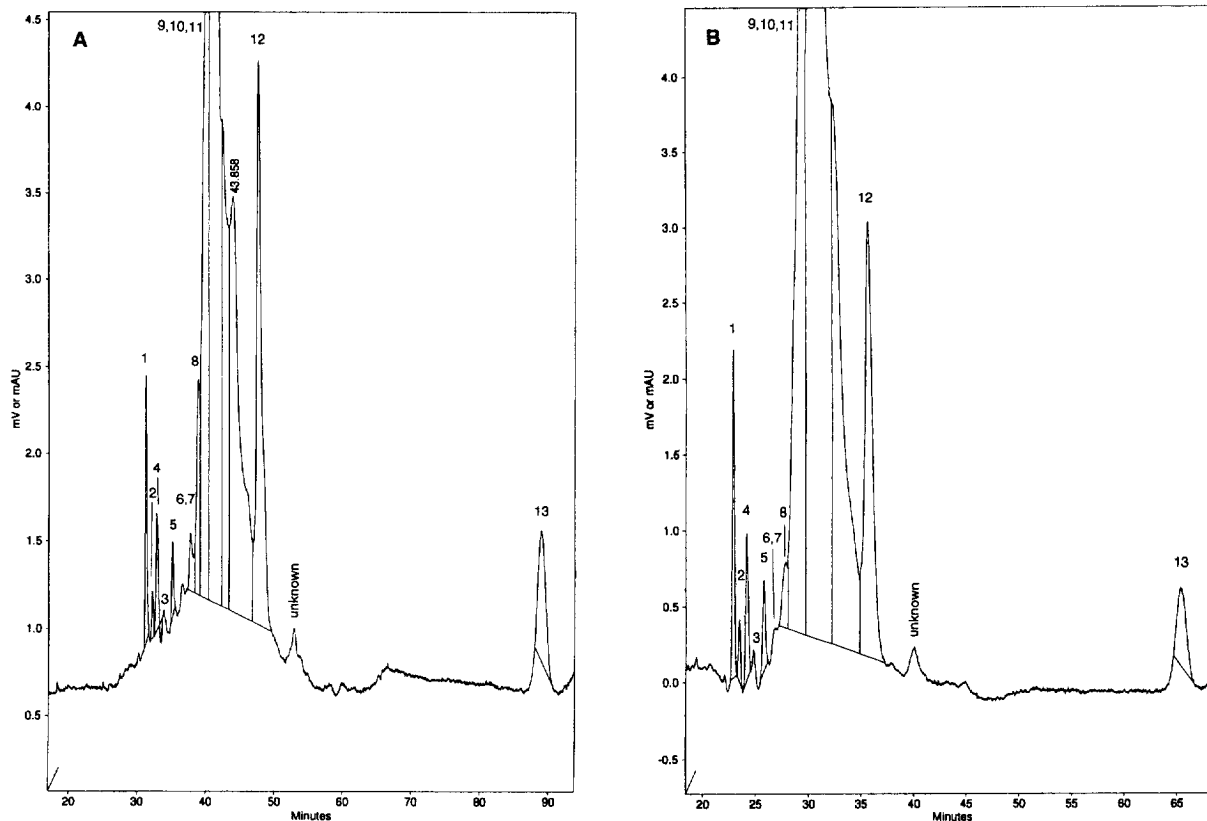


Fig. 2. Separation of collagen type I CNBr peptides obtained from a commercial preparation, spiking and conditions similar to those described in Fig. 1, except that the run was done in a laboratory prepared phosphate buffer pH 2.5 (50 mM) and the voltage was increased to 20 kV per capillary. A=at 25°C and B=at 50°C. Peak identification as for Fig. 1.

mM buffer (plate A); the migration, as expected, was further increased when 100 mM buffer was used (plate B). It is noticeable that in 100 mM background electrolyte the cleavage resistant $\alpha_2(\text{I})\text{CB}_{3,5}$ peptide did not emerge even after 120 min run time.

3.2. Application of the method

Because complete resolution of the marker peptides as described in the previous paragraphs required running times of the order of 60 min and more, our further attempt was to shorten the running time. For this purpose 25 mM buffer (pH 2.5) run at 15 kV and 50°C appeared optimal. A typical run of collagen CNBr peptides obtained from rat tail tendon is presented in Fig. 4. The run time of the small peptides is less than 17 min, although peptides

$\alpha_1(\text{I})\text{CB}_2$ and $\alpha_1(\text{I})\text{CB}_4$ are fused. Their joint peak is clearly resolved from the $\alpha_1(\text{III})\text{CB}_2$ peptide. Because the peak emerging at 20.25 min did not change with additional changes of the CNBr cleavage procedure it was considered pure $\alpha_2(\text{I})\text{CB}_4$ not contaminated with the $\alpha_2(\text{I})\text{CB}_3$ or $\alpha_2(\text{I})\text{CB}_5$ peptide which apparently were recovered in the peak emerging at 30.45 min.

The complete separation of the joint peak of $\alpha_1(\text{I})\text{CB}_2$ and $\alpha_1(\text{I})\text{CB}_4$ from $\alpha_1(\text{III})\text{CB}_2$ is seen in the insert to Fig. 4. Because of the fact that the $\alpha_1(\text{I})\text{CB}_2$ and $\alpha_1(\text{I})\text{CB}_4$ peaks were fused under these conditions and the peptide $\alpha_1(\text{I})\text{CB}_4$ was not available in sufficient quantity and purity, we have decided to use known amounts of CNBr cleaved collagen type I (commercial preparation) for quantitation. In the case of collagen type III the same

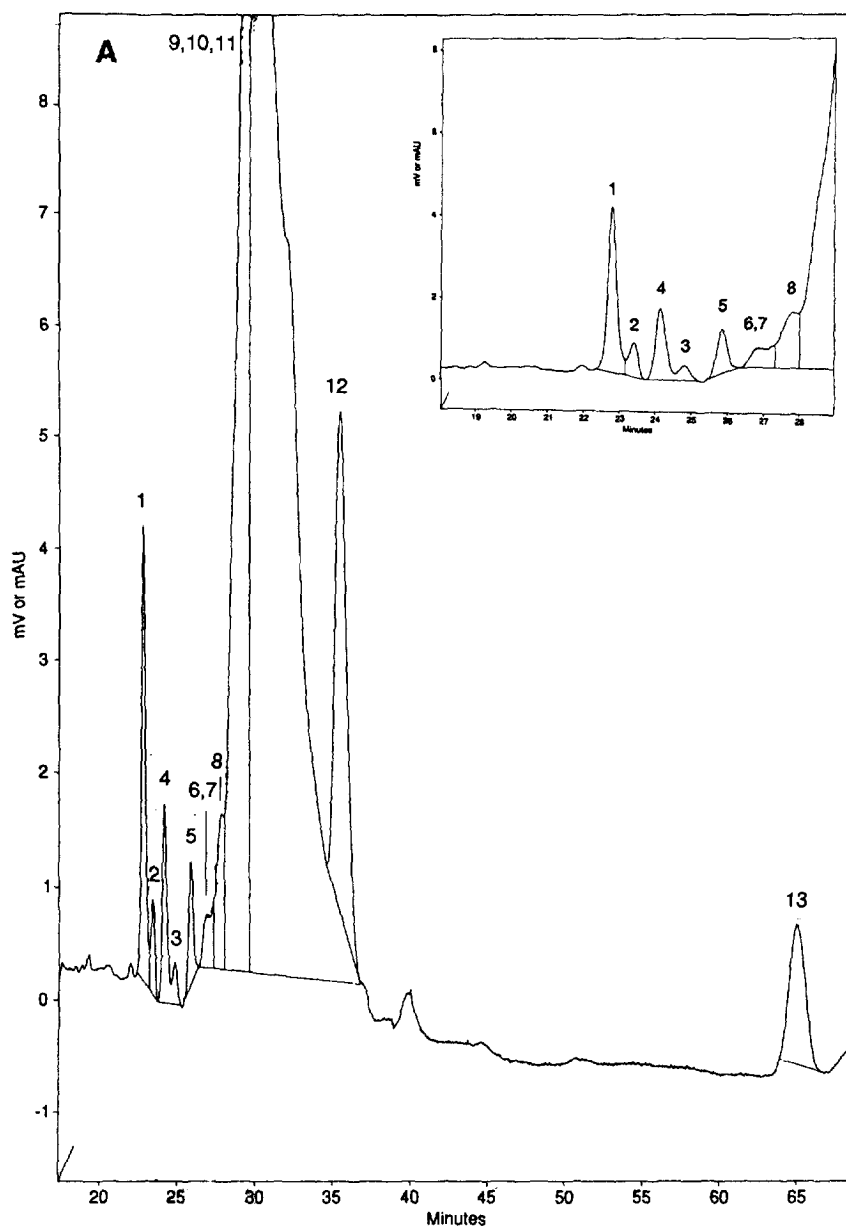


Fig. 3. Separation of collagen type I CNBr peptides obtained from a commercial preparation (see legend to Fig. 1 and Section 2 for details) spiked with marker peptides for collagen type I. Conditions as in Fig. 2, except for buffer concentration, A = 75 mM and B = 100 mM buffer. All runs at 50°C. The $\alpha_2(I)CB_{3,5}$ peak did not emerge even after 120 min running time when the 100 mM buffer was used. Insert: separation of the fast running peptides. Peak identification as for Fig. 1.

approach was used (laboratory prepared collagen type III) along with the isolated $\alpha_1(III)CB_2$ peptide (as described in Section 2). The validity of data obtained by the addition of known amount of

collagen type I hydrolysate to an unknown sample was cross-checked by estimating the amount of collagen type I in a tissue sample based on the change of peak area of the $\alpha_2(I)CB_4$ peptide. Type V

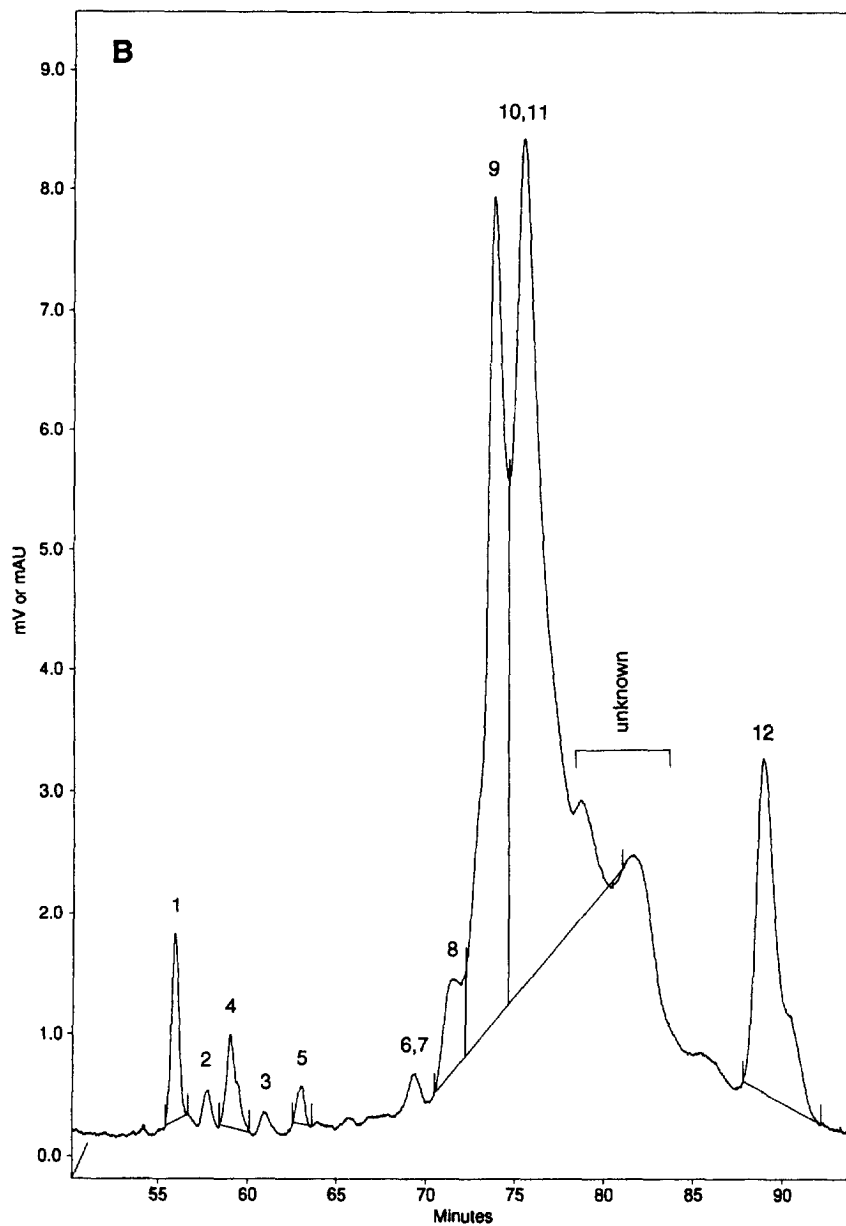


Fig. 3. (continued)

collagen quantitation was based on the standard addition of the $\alpha_1(\text{V})\text{CB}_1$ peptide.

3.3. Calculations

The results of the method presented here are most conveniently expressed as the proportion of a given

collagen type (I, III and possibly V). The most direct approach is based on the area changes of the marker peptides after spiking the unknown sample with a CNBr peptide mixture obtained from known amounts of individual collagen types. Calculation of the proportion of collagen type I was based on measuring the changes of two peak areas, namely the

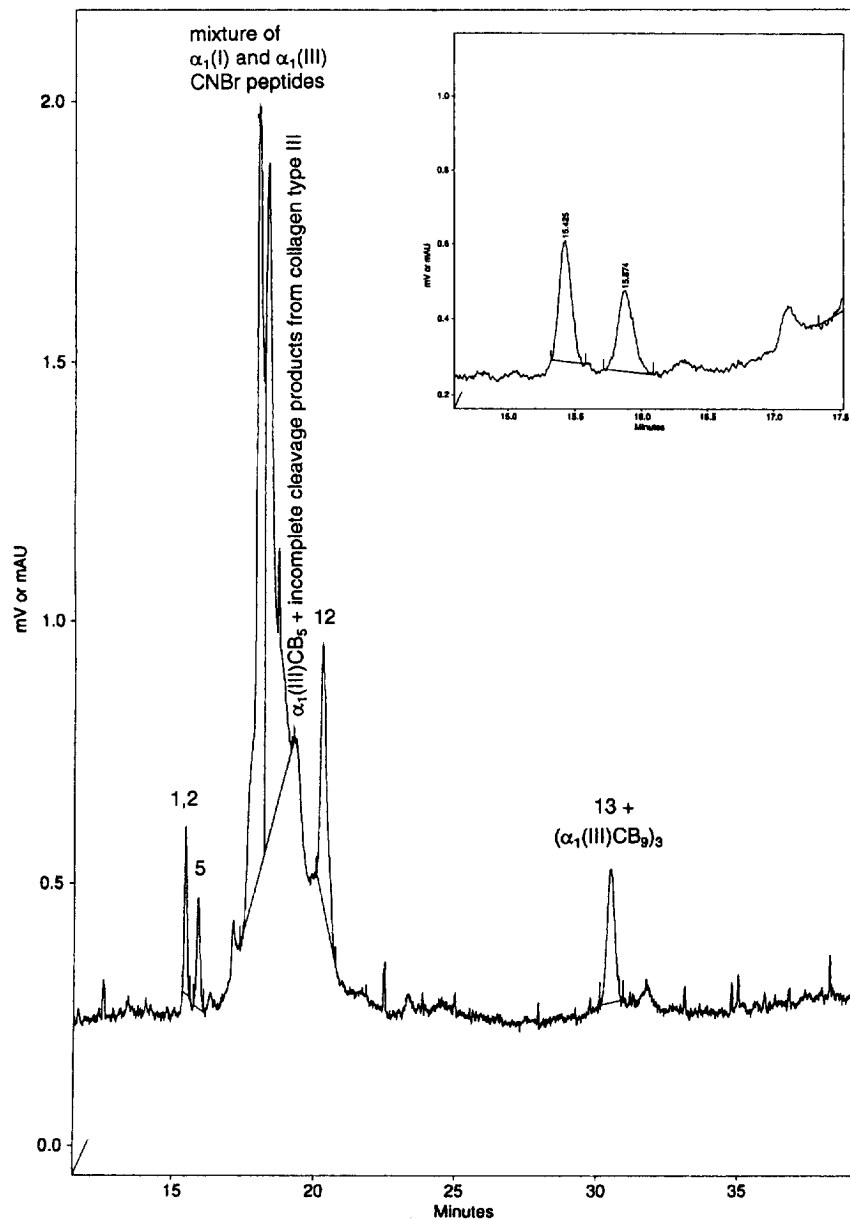


Fig. 4. Separation of collagen CNBr peptides obtained from rat tail tendon; 3 months old Wistar rat, prolonged CNBr cleavage. Run conditions: 25 mM buffer, pH 2.5, 15 kV, 50°C, 200 nm UV detection. Peak identification as for Fig. 1.

change in the fused peak of $\alpha_1(I)CB_2$ and $\alpha_1(I)CB_4$ which was compared with the change of the $\alpha_2(I)CB_4$ peak. Quantitation of collagen type III and V was based on the peak area changes of the marker peptide peaks.

As can be seen from Table 1 either approach

described gave practically the same results. No differences were observed in the final proportion of individual collagen types whether mixtures of known amounts of CNBr peptides of individual collagens were used or if spiking was done by isolated peptides. Spiking with whole mixtures of individual

Table 1
The proportion of collagens type I and III in artificial mixtures

Test mixture	Revealed after spiking with collagen type I and III CNBr peptide mixture			After spiking with $\alpha_1(I)CB_2 + \alpha_1(I)CB_4$ equimolar mixture for collagen type I and $\alpha_1(III)CB_2$ for collagen type III			After spiking with $\alpha_2(I)CB_4$ for collagen type I and $\alpha_1(III)CB_2$ for collagen type III		
	Collagen type I ($\mu\text{g/ml}$)	Collagen type III (%)	Collagen type III ($\mu\text{g/ml}$)	Collagen type I ($\mu\text{g/ml}$)	Collagen type III (%)	Collagen type III ($\mu\text{g/ml}$)	Collagen type I ($\mu\text{g/ml}$)	Collagen type III (%)	Collagen type III ($\mu\text{g/ml}$)
400	0	0	0	430	0	0	420	0	0
300	40	10	54 (± 3)	381 (± 15)	12	38 (± 7)	387 (± 17)	9	42 (± 6)
320	80	20	75	308	19	87	296	22	89
280	120	30	134	285	32	122	280	29	128
240	160	40	155	262	39	158	259	37	165
200	200	50	197 (± 12)	187 (± 21)	50	203 (± 18)	179 (± 23)	52	204 (± 12)

Numbers in parentheses represent \pm S.D. ($n=5$).

purified collagen types was tested because it simplifies the procedure considerably; what is really needed are pure standards of collagen type I, III and V. Because obtaining (non-contaminated) pure samples of collagen types III and V is a tedious business, more complex experimentation is needed for isolating the marker peptides. It is surprising that standard addition of purified peptides offered practically the same results as standard addition of the whole mixture because it is quite likely that the peptide isolated could be contaminated (e.g. by salts) from the preceding chromatographic steps.

The minute amounts of uncontaminated peptides that could be obtained almost prevent the construction of a calibration graph. Applicability of the proposed procedure is shown in Table 2 where our own results obtained by standard addition method (of both purified marker peptides and mixtures of CNBr peptides obtained from a known amount of a particular collagen type) are compared with literature data obtained by various methods and other authors. Our results fit well with those obtained by Miller et al. [16]. The only step in which the present procedure is different from that of Miller et al. is the final separation, done by gel chromatography in [16] and by capillary electrophoresis in our case. On the other hand, in the paper of Macek et al. [25] the estimation of the percentage of collagen type III in rat skin was obtained by fraction precipitation of pepsinized insoluble collagens and still the results are in agreement with our data.

We agree with the consideration of Miller et al.

[16] that the most convenient method of expressing the data is the determination as the proportion of a given collagen in the total type I, III and V collagen pool. Rather than calculating the total area for the peaks representing type I, III and V according to the equation

$$A_T = (A_I \cdot 1.5) + A_{III} \cdot 0.9 + A_V \quad (1)$$

(where A_T is total area, A_I , A_{III} and A_V areas of individual marker peptides) we preferred direct estimation by the standard addition method. The rationale here was twofold: the Miller et al. [16] approach requires the introduction of correction factors, namely 1.5 for the area of type I collagen [which accounts for the presence of the marker peptide $\alpha_1(I)CB_2$ in only two of the three chains of collagen type I] while the factor 0.9 should respect the fact that the marker peptide for type III collagen $\alpha_1(III)CB_2$ contains 40 amino acid residues (collagen type V marker has only 36 amino acid residues) (see also Section 4).

4. Discussion

Looking at the CNBr peptide profiles it is obvious that there are extreme differences in the area occupied by individual peaks. Some of the peaks do not represent a single peptide, nevertheless if one considers the stoichiometry of CNBr fragments (considering e.g. collagen type I) all the peptides present should be either in the 1:1 or 2:1 molar ratio

Table 2
The proportions of fiber-forming collagen (type I, III, and V in tissue samples)

Specimen	Experimental data			Literature data			Reference
	Type I	Type III	Type V	Type I	Type III	Type V	
Rat skin collagen ^a	62	38	ND	64	36	ND	[25]
Placenta membranes (human)	75	20	5	70	27	3	[16]
Left circumflex coronary artery (human)	80	20	ND	82	18	ND	[16]
Thoracic aorta (human)	59	41	ND	62	38	ND	[16]
Abdominal aorta	52	48	ND	47	53	ND	[16]
Rat tail tendon ^b	57	43	ND	—	—	—	[16]
Rat peripheral pulmonary arteries	58	42	ND	74	26	ND	[28]

^a Rats 17 months of age.

^b Rats 6 months of age.

ND=not detected.

depending on their parent polypeptide chains. Though the actual absorbance maximum of the UV bond is about 185 nm and our measurements were done at 200 nm, it seems reasonable to assume that a protein molecule which contains many more peptide bonds than a small peptide would absorb proportionally a larger amount of UV light precluding any change in absorbance due to the presence of cyclic imino acid (proline and hydroxyproline) or the formation of secondary structures that might alter UV absorption. This assumption was recently proven to be true for small, aromatic ring devoid peptides [26] and appears to be applicable to some of the CNBr released collagen peptides, and was shown indirectly by the results of Miller et al. [16]. However, there is some indication that collagen type III released peptides have a tendency for higher UV absorbance compared to type I counterparts of similar molecular mass [27]. To what extent this is caused by differences in the distribution of proline and hydroxyproline and, perhaps, phenylalanine remains to be elucidated.

It is observed here that the peptides are generally eluted according to their increasing molecular mass (see also Ref. [17]). This, however, was proven for collagen type I arising peptides only and applies for peptides of containing more than 149 AA residues. However, this dependence is different for collagen type I and III peptides; the latter appear to migrate slightly faster with the electroosmotic flow and in a tissue collagen analysis are hidden in the dominant double peak; they cause its shifting but they still leave the $\alpha_2(\text{I})\text{CB}_4$ peptide well accessible for quantitation.

From the practical point of view there are several points worth mentioning. First, it can happen that sticking of collagen CNBr peptides is excessive and that during the run not all (particularly the large) peptides are eluted. We have never seen this happen with the small (marker peptides) though we did see a loss of recovery in such a situation of the $\alpha_2(\text{I})\text{CB}_4$ peptide. It is therefore recommended to apply the following washing steps: 5 min, run buffer, 2 min, 0.1 M H_3PO_4 , 7 min, run buffer all at 50°C.

An easy indication of excessive sticking of the peptides to the capillary wall is current instability. It is always advisable to let the electrophoresis run for about 1 h after every three analyses. It was also

observed that sticking is more intensive with a capillary that has been used for a long time. It is therefore recommended to exchange the capillary after 20–30 runs, because even repeated washing, transferring the capillary into the alkaline media (0.1–1 M NaOH) and washing with highly concentrated detergent (500 mM SDS) need not necessarily reestablish the separation capacity of the capillary column. Such distorting effects, however were never seen during the first 10 runs with a new capillary.

5. Conclusions

It has been demonstrated that collagen type I, III and V proportions in tissues can be assayed by solubilizing tissue collagen by extended CNBr treatment followed by the separation of the released peptides. Quantitation (establishing of individual collagen types proportion) was done by using the standard addition method of relatively low-molecular-mass peptides ($\alpha_1(\text{I})\text{CB}_2$ and $\alpha_1(\text{I})\text{CB}_4$ for collagen type I, $\alpha_1(\text{III})\text{CB}_2$ for collagen type III and $\alpha_1(\text{V})\text{CB}_1$ for collagen type V). The results obtained for collagen type I can be verified in the same run by establishing the area of $\alpha_2(\text{I})\text{CB}_4$ peptide which runs after the main combined peptide peak but before the peak of the incompletely cleaved $\alpha_2(\text{I})\text{CB}_{3,5}$ peptide. The $\alpha_2(\text{I})\text{CB}_4$ peptide may be contaminated with $\alpha_2(\text{I})\text{CB}_3$ and $\alpha_2(\text{I})\text{CB}_5$, if the peptide bond joining these two peptides is cleaved, however under the conditions specified they remained uncleaved and run with a much longer migration time (as compared to $\alpha_2(\text{I})\text{CB}_4$). Quantitation was preferably done by the standard addition method using CNBr peptide mixtures obtained from known amounts of purified individual collagen types. The fact, that such standards can be used for assaying fiber-forming collagen types (I, III and V) in tissues overcomes the need of using purified peptide standards, the preparation of which is a tedious operation. Though the peptides $\alpha_1(\text{I})\text{CB}_2$ and $\alpha_1(\text{I})\text{CB}_4$ can be baseline separated by selecting appropriate run conditions, in routine analyses we have used their joint peak and compared it with the data obtained from the $\alpha_2(\text{I})\text{CB}_4$ peak area measurement.

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